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The Spectrin-Ankyrin Skeleton Controls CD45 Surface Display and Interleukin-2 Production

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Summary

With T cell receptor stimulation, intracellular pools of CD45 and spectrin move to the surface. These processes are coupled. In both peripheral lymphocytes and Jurkat T cells, βI spectrin and ankyrin associate with CD45. In Jurkat T cells, βI spectrin peptides suppress surface recruitment of CD45 and CD3 and abrogate T cell activation. Other glycoproteins such as CD43 are not altered by the spectrin peptides. Spectrin's effects are mediated by ankyrin, which binds directly to the cytoplasmic domain of CD45 ($K_d = 4.3 \pm 3.0$ nM). These data reveal a novel and unexpected contribution of the spectrin-ankyrin skeleton to the control of T lymphocyte function.

Introduction

CD45 is a membrane phosphotyrosine phosphatase expressed in cells of hematopoietic lineage. Its genetic deletion causes defective thymocyte development and failed receptor-mediated activation (Wallace et al., 1997), while defects in CD45 underlie human forms of severe combined immunodeficiency (Buckley, 2001). Proximity of CD45 to the T cell receptor (TCR) is necessary for activation (Turka et al., 1992), but the mechanism by which CD45 modulates TCR function remains a topic of interest and controversy (Alexander, 2000; Leitenberg et al., 2001). Available data suggest that CD45 may prime the src family tyrosine kinases required for TCR signaling by removing an inhibitory phosphate (Ledbetter et al., 1993), an action requiring the formation of macromolecular protein complexes on the membrane that include CD45, the TCR, and other costimulatory molecules (Ishii et al., 2001; Turka et al., 1992). Spatial segregation between CD45 and other receptors may also be effected by its exclusion from detergent insoluble glycolipid (DIG) rafts (Janes et al., 1999), sphingomyelin-enriched clusters harboring the TCR. While many details remain to be clarified, these observations suggest that key determinants of the functional activity of CD45 and its relationship to the TCR include processes that control the surface display and surface partitioning of CD45 between laterally organized microdomains.

In vertebrate cells, an extensive membrane-associated spectrin-ankyrin scaffold tethers membrane and cytosolic proteins to the actin and microtubule cytoskeleton. This skeleton controls the lateral organization and stability of many surface proteins and also participates in the organization and dynamics of internal membrane compartments, including the Golgi, endosomes, and acidic phospholipid axonal vesicles, and with vesicular traffic in the secretory pathway (Beck and Nelson, 1998; De Matteis and Morrow, 2000; Muresan et al., 2001). At least two regions of β spectrin mediate its association with organelles: (1) an NH_2-terminal region that includes a direct membrane association domain (MAD1) and an actin and ARP1 binding domain; and (2) a region near the COOH terminus that includes a pleckstrin homology (PH) domain that binds directly to acidic phospholipids such as membrane phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) (De Matteis and Morrow, 2000). Linkage to integral membrane proteins is mediated predominantly by ankyrin, which also binds to β spectrin's 14–15th repeat region. Multiple isoforms of spectrin exist, generated by seven distinct genes and by extensive alternative mRNA splicing (De Matteis and Morrow, 2000). βI spectrin has been definitively identified only in erythrocytes, brain, and muscle, although immunocross-reactive forms have been found in the Golgi of other cells. βII spectrin is widely expressed and generally associated with the plasma membrane (PM). βIII spectrin associates with both the PM as well as with the Golgi in neurons and epithelial cells, and with actively transported vesicles in the secretory and endocytic pathways (Holleran et al., 2001; Muresan et al., 2001; Sakaguchi et al., 1998; Stankewich et al., 1998). βIV and βV spectrin are specialized forms with more restricted tissue distributions. Under most conditions, β spectrins join with an α-spectrin to form an αβ heterodimer. αI spectrin is found predominantly in red cells; αII spectrin is widely distributed in most other cells.

Lymphocytes contain, in addition to PM-associated spectrin, an unusual cytoplasmic skeleton composed of a spectrin-ankyrin-rich perinuclear vesicular aggregate (Black et al., 1988). This complex is variably present in both cultured and native lymphocytes (Gregorio et al., 1994; Pauly et al., 1986), is regulated in part by Ca^{2+} and PKC (Gregorio et al., 1993, 1992), and is recruited to the PM coincident with cell activation (Gregorio et al., 1992; Masso-Welch et al., 1999). However, the precise composition or role of this spectrin-ankyrin-rich organelle remains uncertain. Given the newly recognized association of spectrin with both Golgi and endosomal compartments, it seems likely that this perinuclear aggregate in lymphocytes represents a modified and expanded trans-Golgi network (TGN) or a late endosomal compartment, presumably containing nascent or recycled membrane constituents poised for recruitment to the membrane upon cell activation. In either case, these early observations point to a role for spectrin and ankyrin in the biogenesis of the lymphocyte membrane and its response to TCR stimulation.

In the present study, we have evaluated spectrin's composition, distribution, and function in lymphocytes and its relationship to T cell function and CD45 dynamics. We identify two spectrins not previously observed in lymphocytes and demonstrate that βI spectrin, acting

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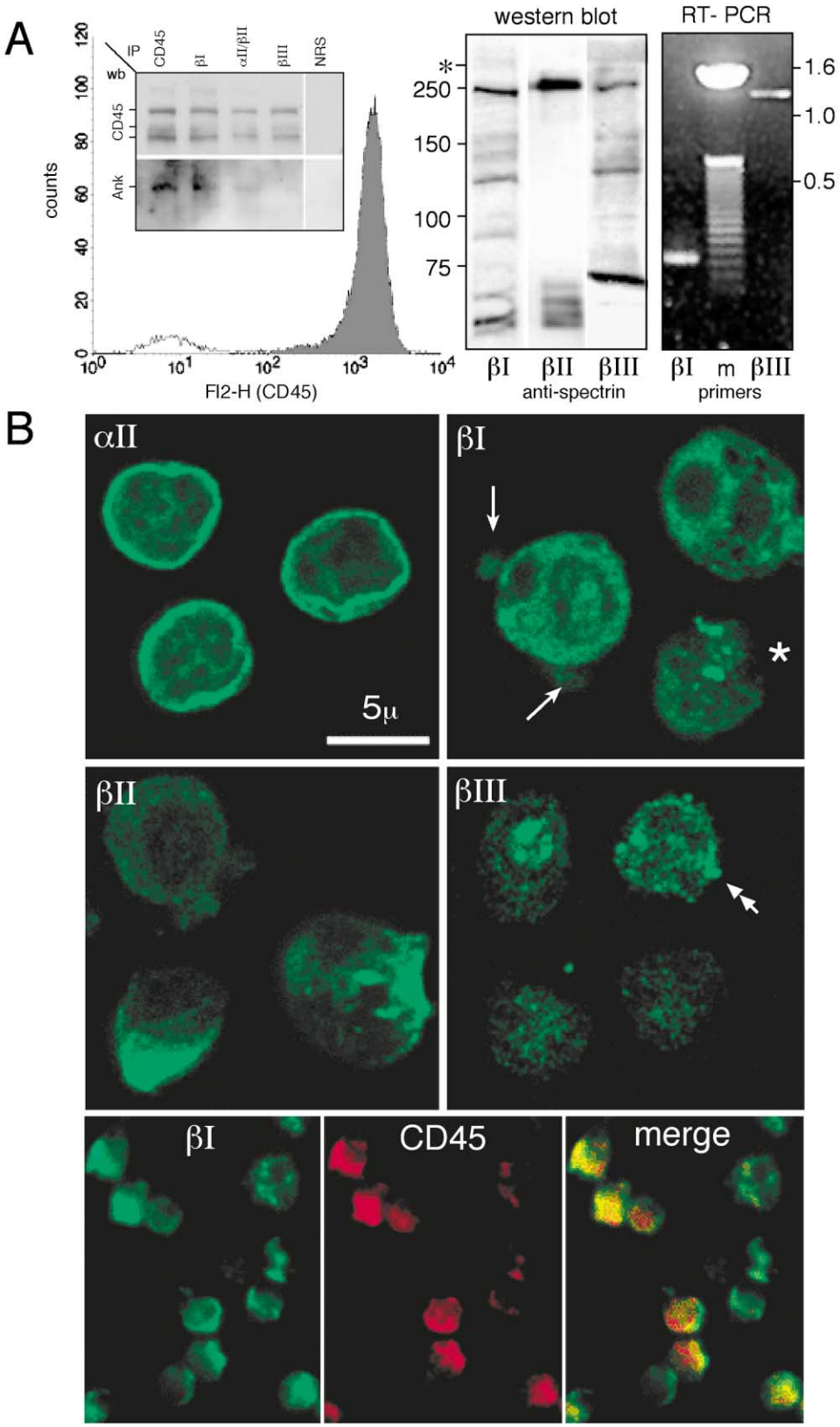


Figure 1. Primary Lymphocytes Express Multiple Spectrins that Associate with CD45

(A) Flow cytometry of fresh purified human PBLs was composed of CD45-positive lymphocytes (72%), with minor contamination by monocytes (10%). Dead cells and cellular debris comprised less than 10% of the preparation. Western blot analysis of the CD45-enriched lymphocyte fraction revealed βI, βII, and βIII spectrin isoforms. A faint secondary band at ≈276 kDa (*) represents the βIΣ2 isoform. RT-PCR analysis

with ankyrin, facilitates the movement of CD45 and CD3 to the lymphocyte surface. Disruption of this process abrogates the ability of T cells to be activated. These findings reveal a role for spectrin and ankyrin in the signal transduction pathways of T lymphocytes and suggest novel mechanisms by which the display and surface partitioning of key lymphocyte receptors and costimulatory molecules can be regulated.

Results

CD45 Associates with Spectrin and Ankyrin in Peripheral Blood Lymphocytes

Independent studies have detected spectrin at the membrane and in cytoplasmic aggregates of lymphocytes, and have shown that CD45 can bind directly to spectrin in vitro (Lokeshwar and Bourguignon, 1992). To understand whether CD45 binds to the spectrin and ankyrin skeleton in vivo, a CD45-rich, monocyte-depleted population of fresh human peripheral blood leukocytes (PBL) were evaluated by immunoprecipitation and Western blotting with anti-CD45 or with antibodies specific for the different spectrins (Figure 1A). The antibody to CD45 revealed bands in Western blots ranging from 220 to 180 kDa. The CD45 and βI spectrin precipitates were rich in ankyrin, indicating that ankyrin was part of the CD45-spectrin complex. Antibodies to each of the spectrins also precipitated CD45, suggesting the presence of two novel spectrins (βI and βIII) previously unrecognized in lymphocytes, and the broad association of CD45 with several spectrin compartments. The presence of βI and βIII spectrin in PBLs was directly confirmed by Western blot and by RT-PCR (Figure 1A, right panels). Ankyrin was relatively enriched in the CD45/βI spectrin compartment versus the other spectrin precipitates.

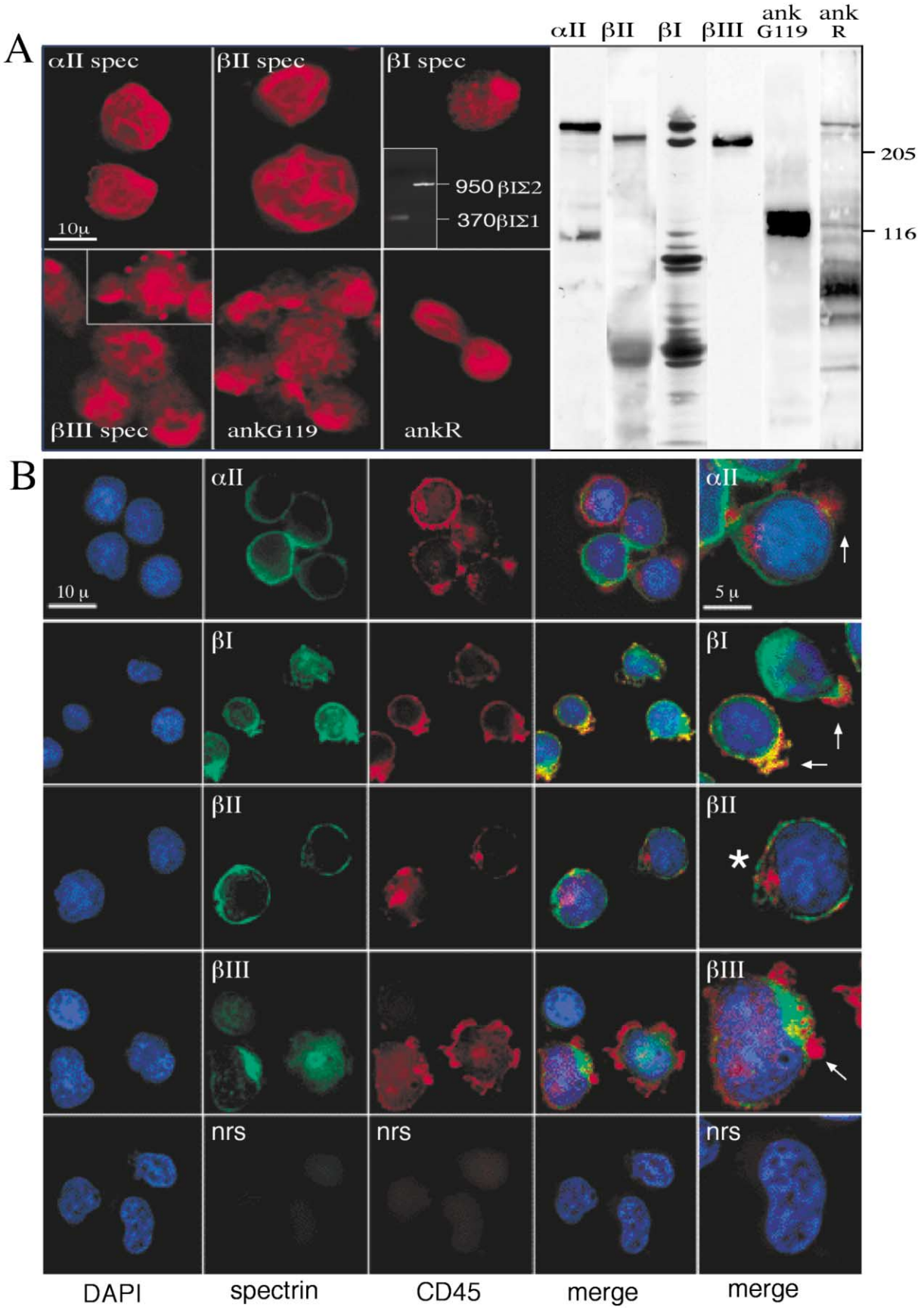
Given the surprising finding of multiple spectrin isoforms in PBLs, it was of interest to determine their intracellular disposition. Indirect immunofluorescence revealed for each spectrin a somewhat variable and overlapping, but distinctive distribution (Figure 1B). αII spectrin was uniformly distributed on the PM, as is typical of other cells; small cytoplasmic accumulations were also evident, presumably due to its association with the different β spectrins. βII spectrin also displayed a surface membrane distribution, albeit less pronounced than with αII spectrin. In other cells, it was found concentrated in relatively large surface patches near one end of the cell, similar to the variable and polarized spectrin distribution noted in earlier studies, and occasionally but not consistently was faintly present on surface blebs. βI spectrin displayed a largely cytoplasmic and patchy surface distribution and was the spectrin most often accumulated in protruding surface blebs visible on many of the lymphocytes (Figure 1B, arrows). This spectrin also appeared to concentrate in cytoplasmic aggregates, presumably near the nucleus, in a pattern similar to that previously observed in lymphocytes with avian spectrin antibodies (Black et al., 1988). Antibodies specific for either of two alternative transcripts of βI spectrin, termed βIΣ1 and βIΣ2, as well as antibodies that were pan-reactive to all βI isoforms, gave indistinguishable results, suggesting the presence of both βIΣ1 and βIΣ2 spectrin in these cells. The pattern for βIII spectrin was similar to that observed in epithelial cells (Stankewich et al., 1998), in that it stained coarse cytoplasmic vesicles, with a putative juxtanuclear concentration, along with focal areas of the surface membrane (Figure 1B, double arrows). Finally, because of the strong association between CD45 and βI spectrin detected by immunoprecipitation, their distribution was examined by double indirect immunofluorescence (Figure 1B, bottom panel). Substantial (although not complete) overlap of these proteins was noted, particularly in surface blebs, polarized surface patches, and within intracellular accumulations.

βI Spectrin Codistributes with CD45 in Jurkat T Cells

The human Jurkat T lymphocyte line offers an experimentally accessible system useful for the evaluation of spectrin perturbation on the dynamics of CD45 trafficking. Immunofluorescent and Western blot analysis confirmed in this line the same complex profile of spectrin isoforms as in PBLs (Figure 2A). In Jurkat cells, both αII and βII spectrin associated predominantly with the PM, often concentrating on surface folds, a pattern similar to that observed in PBLs. βI spectrin was found in the cytoplasm, in dense perinuclear aggregates, and in polarized surface patches and surface blebs on the PM (also Figure 2B). Again, this pattern was reminiscent of that observed in PBLs. βIII spectrin was usually present as coarse cytoplasmic punctate structures concentrated over the putative Golgi region; in some cells, it was also concentrated into small dense surface blebs, like the occasional small βIII spectrin blebs observed in PBLs. Ankyrin G119 ($AnkG_{119}$), a short form of ankyrin associated with the Golgi (Devarajan et al., 1996), displayed a pattern similar to βIII spectrin. Ankyrin R was present on the PM and often polarized in dense membrane patches. Western blot analysis confirmed the presence of these proteins at their expected sizes. Two

confirmed the presence of both βI and βIII transcripts in PBLs. (Inset) The association of CD45 with elements of the spectrin-ankyrin cytoskeleton was assayed by immunoprecipitation. Antibodies to each of four spectrins (βI, αII/βII, βIII) efficiently coimmunoprecipitated CD45. Antibodies to CD45 also coprecipitated a high-molecular weight ankyrin (Mr ≈ 215 kDa) that was reactive with AnkR antibodies. This same ankyrin was equally well precipitated by βI spectrin antibodies and to a lesser degree by αIIβII and βIII spectrin antibodies.

(B) Indirect immunofluorescent analysis of human PBLs demonstrated overlapping but distinct patterns of spectrin staining for each of the spectrin isoforms. αII spectrin was most abundant on the PM. βII spectrin was present on surface folds of the PM and was often concentrated in relatively large surface patches. βI spectrin displayed a cytoplasmic and patchy surface distribution and was the spectrin most often accumulated into the protruding surface blebs visible on many of the lymphocytes (arrows). This pattern was similar whether antibodies to βIΣ1 or βIΣ2 were used (βIΣ2 results shown). Spectrin βI also appeared to concentrate in an aggregate near the nucleus, in a pattern similar to that previously observed in lymphocytes with avian spectrin antibodies (see text). βIII spectrin displayed a coarse vesicular cytoplasmic pattern with focal areas of intense surface membrane staining (double arrows).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merge

βI spectrin bands (Mr ≈220 and ≈276 kDa) were observed, corresponding to the βIΣ1 and βIΣ2 isoforms as confirmed by RT-PCR (Figure 2A, inset). The smaller bands in each lane presumably represented minor degradation products. The cellular distribution of the spectrins was then compared with CD45 using deconvolution microscopy (Figure 2B). At the PM, CD45 was often concentrated into surface patches and surface blebs (Figure 2B, arrows). Occasional internal pools of CD45 in a juxtanuclear position were also noted (Figure 2B, *). Spectrin βI was the predominant isoform codistributed with the surface patches and blebs of CD45.

βI Spectrin Peptides Disrupt the Surface Display of CD45

To better understand the relationship between βI spectrin and CD45 organization, Jurkat cells were transfected with βI spectrin constructs encoding specific functional domains. Three peptides were studied. Given the coprecipitation of CD45 with spectrin and ankyrin, as well as the sensitivity of lymphocyte activation to agents that disrupt or modify the actin skeleton (Dustin and Cooper, 2000), the two functional domains of greatest interest were the actin/ARP1 binding domain (ABD) and the ankyrin binding domain (Figure 3). In earlier work, transfection of a βI spectrin peptide ($\beta I_{N\text{-}5}$) encompassing the ABD into epithelial cells led to dominant suppression of the transport of α-Na,K-ATPase through the secretory pathway (Devarajan et al., 1997). A second peptide chosen for study was $\beta I_{14\text{-}15}$, which encompasses the ankyrin binding domain in repeats 14–15. The $\beta I_{N\text{-}5}$ peptide will bind actin and dynein/dynactin complexes but will not support ankyrin-dependent linkages between membrane proteins and spectrin. The $\beta I_{14\text{-}15}$ peptide will compete with endogenous spectrin for ankyrin-dependent binding to membrane proteins but will not couple these proteins to other cytoskeletal elements such as actin, dynein/dynactin, or to other spectrins. A third peptide was also prepared, $\beta I_{N\text{-}5,15}$, that included the ABD and ankyrin binding domain, but deleted spectrin's other functions, including the binding of membrane phosphatidylinositol phospholipids via its PH domain (Godi et al., 1998).

At least five stable independent cell lines were established for each construct. All gave comparable results. Only low levels of stable βI spectrin peptide expression could be achieved, presumably because higher levels were cytotoxic. The stable lines that were established expressed each construct with fidelity as measured by RT-PCR (Figure 3, top), and in approximately equal protein amounts as measured by flow cytometry in permeabilized cells with antibodies specific to the FLAG epitope (Figure 3). Immunofluorescent microscopy revealed a marked loss of surface CD45, particularly in the surface blebs, for cells expressing $\beta I_{N\text{-}5}$ and $\beta I_{14\text{-}15}$. These cells also appeared to accumulate increased cytosolic concentrations of CD45 as dense cytosolic aggregates (Figure 3). Conversely, in cells expressing $\beta I_{N\text{-}5,15}$ the surface display of CD45 approximated normal levels, and many cells even appeared to have a super abundance of CD45-positive surface patches and blebs (Figure 3). No significant changes in the endogenous spectrin distributions in these cells were detected.

The surface levels of CD45 were also assessed by FACS analysis (Figure 4). These results paralleled the immunofluorescent observations and confirmed that the $\beta I_{N\text{-}5}$ and $\beta I_{14\text{-}15}$ spectrin peptides reduced surface levels of CD45 by over a log, while the $\beta I_{N\text{-}5,15}$ peptide was without significant effect. Western blots revealed no significant change in total cellular CD45, regardless of the spectrin peptide expressed (Figure 4B), indicating that the βI spectrin peptides affected only the surface distribution and surface display of CD45 but not its net rate of synthesis, degradation, or shedding from the surface. Similar experiments were also carried out with transfected βIII spectrin constructs (Figure 4A). While the spectrins are a closely related gene family, considerable sequence divergence exists within this family, presumably accounting for their often unique intracellular distributions and functions. Pertinent to the present study, the degree of sequence homology (βI versus βIII) was: $\beta_{N\text{-}5}$, 61.5%; $\beta_{14\text{-}15}$, 54%; $\beta_{N\text{-}5,15}$, 55.0%. Within just the ankyrin binding domain (repeat 15), the homology between βI and βIII spectrin drops to 41.5%. Thus, these proteins are substantially different. Accordingly, even though βIII spectrin partially colocalized and coprecipitated with CD45 (although it bound little ankyrin, Figure 1), it had little effect on the surface abundance of CD45 (Figure 4). Thus, the inhibitory effects of these peptides on CD45 surface display appeared to be specific for βI spectrin constructs.

βI Spectrin Peptides Reduce Surface CD3 but Not Surface CD43 and Block T Cell Activation

The impact of the transfected βI spectrin peptides on the surface display of other selected receptors was also

Figure 2. βI Spectrin Codistributes with CD45 in Jurkat T Cells

(A) Jurkat T cells were examined by indirect immunofluorescence for the presence of different spectrins. αII and βII spectrin were found predominantly on the PM. βI spectrin was present as perinuclear cytoplasmic aggregates or on surface blebs (also see [B]). βIII spectrin usually displayed a coarse punctate perinuclear distribution, as did Ank_{G119}. Occasional cells also displayed small intense βIII spectrin-enriched knobs on their surface, very similar to a pattern occasionally observed in PBLs. Ank_R displayed a distribution similar to βI spectrin and was also present on the PM, often in polarized surface patches. Western blots again confirmed the presence of each of the spectrins. Note the presence of two large βI reactive bands, indicative of βIΣ1 (≈220 kDa) and βIΣ2 (≈276 kDa). RT-PCR analysis confirmed that both βIΣ1 and βIΣ2 transcripts were expressed.

(B) Deconvolution immunofluorescent microscopy of CD45 (red), spectrin (green), and their relationship to the nucleus (stained with DAPI, blue). Each deconvoluted image represents an ≈1 μm slice though the cell. CD45 codistributes predominantly in surface blebs (arrows) with βI spectrin, as indicated by the areas of overlapping fluorescence (yellow). There is less association of CD45 with βIII spectrin, and essentially no codistribution with αII or βII spectrin. Also apparent in some cells is a tight peri-nuclear concentration of CD45 (marked with an *). The right-most column represents a collage of cells presented at higher magnification (note scale). Negligible immunofluorescence was noted with preimmune serum (NRS).

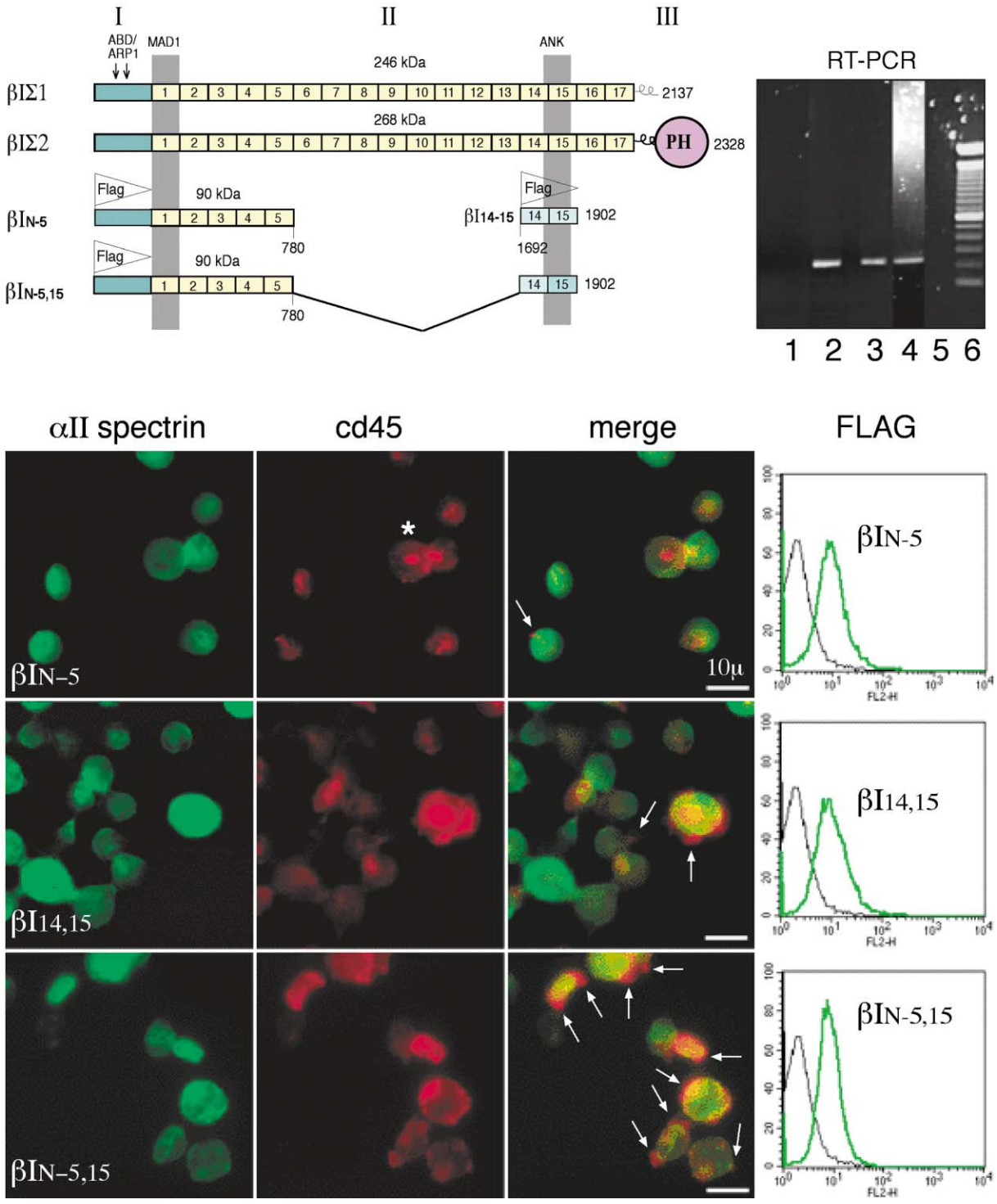

Figure 3. Transfection of βI Spectrin Peptides into Jurkat Cells

(Top) Cartoon depicting the relationship of the peptides encoded by the three βI constructs to βI spectrin. The loci of the actin/ARP1 binding domain near the NH_2 terminus, the MAD1 membrane binding region in repeat 1, and the ankyrin binding domain in repeats 14–15 are depicted. All constructs encoded a FLAG epitope tag at the NH_2 terminus to enable their detection in transfected cells. Analysis by RT-PCR of each cell line confirmed the expression of the appropriate construct. The cell lines analyzed in each lane were: lane 1, vector; lane 2, $\beta I_{N\text{-}5}$; lane 3, $\beta I_{14\text{-}15}$; lane 4, $\beta I_{N\text{-}5,15}$; lane 5, wt cells; lane 6, markers. Note that in each case, primers were chosen that required the presence of both FLAG sequence and the appropriate βI spectrin sequence.

(Bottom) Impact of the transfected peptides on the surface distribution of CD45 (red). Also shown is the distribution of αII spectrin (green), along with a merged image for each transfected cell line. All stable Jurkat lines expressed similar levels of transfected construct, as measured by flow cytometry (FLAG, right panel). Note that in cells expressing $\beta I_{N\text{-}5}$ or $\beta I_{14\text{-}15}$, there is a reduction in the quantity and intensity of CD45 associated with the surface membrane, and especially with surface membrane blebs, compared to wild-type cells (Figure 2). These cells also appear to accumulate increased cytosolic concentrations of CD45, appearing as dense perinuclear cytosolic aggregates (*). Conversely, in cells transfected with $\beta I_{N\text{-}5,15}$ there is enhanced abundance of CD45 associated with the surface and with surface membrane blebs.

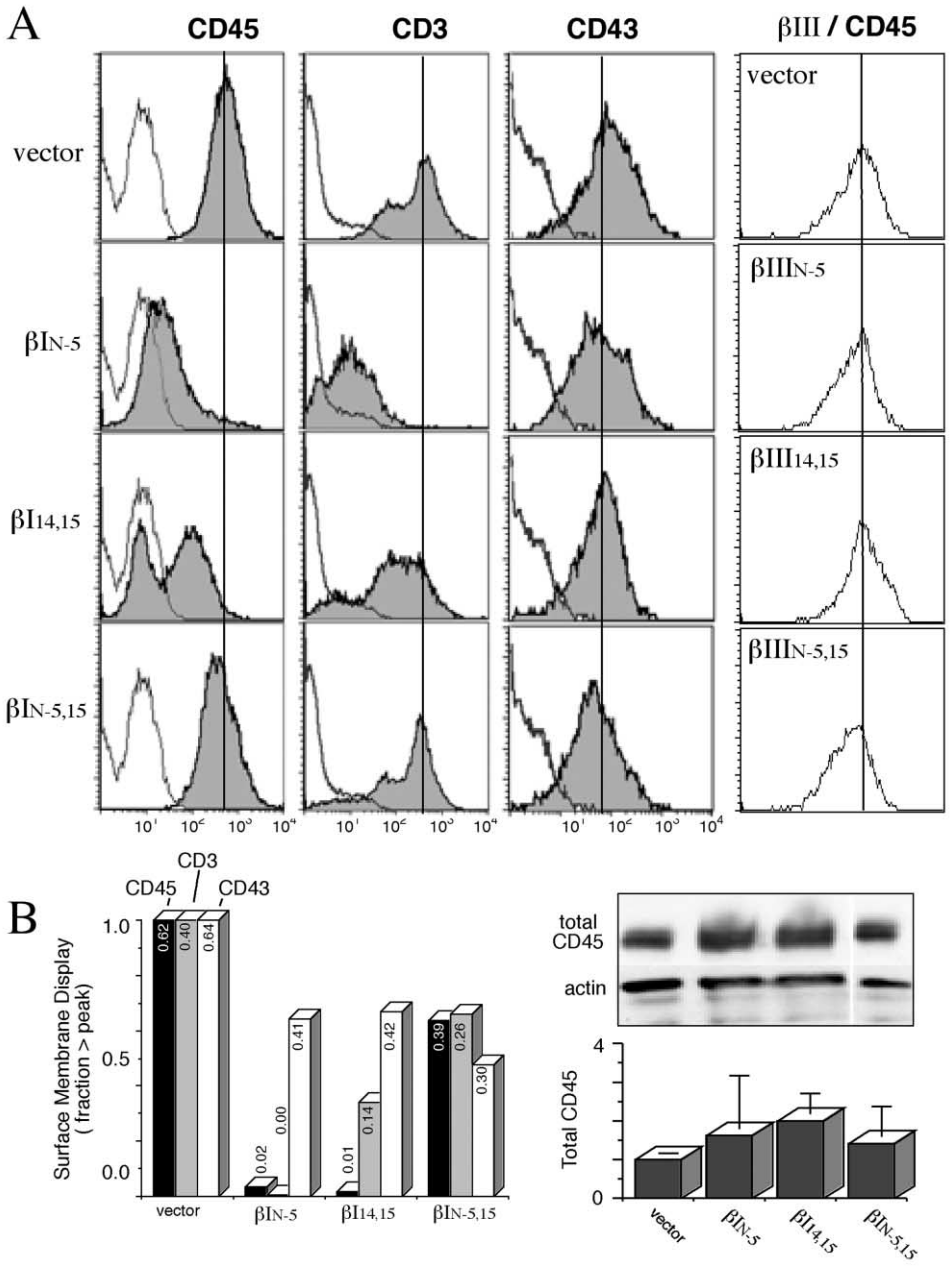

Figure 4. Spectrin Peptides Suppress the Display of Only a Subset of Membrane Proteins

(A) Flow cytometry reveals suppression of surface CD45 and CD3 in Jurkat cells expressing the βI_{N-5} and βI_{14-15} constructs. These constructs have no effect on the surface display of CD43, a membrane glycoprotein linked to the cortical actin skeleton by moesin (Allenspach et al., 2001). Similar constructs derived from βIII spectrin (labeled here with GFP) do not suppress CD45. (Separate experiments have established that the FLAG- or GFP-labeled constructs perform similarly).

(B) The suppression of CD45 and CD3 by βI spectrin peptides is substantial, as measured by the fraction of cells expressing on their surface more than the median level expressed by control cells.

(C) The suppression of surface display is not due to a suppression of CD45 synthesis or to accelerated degradation or shedding, since total cellular levels of CD45 remain constant (n = 3 independent determinations, ±2SD).

evaluated by FACS analysis (Figure 4). Another protein that responded in a manner analogous to CD45 was CD3, a component of the TCR complex that may also interact with CD45 (Leitenberg et al., 2001). The surface level of CD3 was reduced by over a log in cells expressing βI_{N-5} or βI_{14-15} spectrin but not in cells carrying $\beta I_{N-5,15}$. Conversely, a different lymphocyte membrane glycoprotein, CD43, was unaffected by any of the spectrin peptides. Thus, the spectrin-dependent suppression of receptor display was specific for both the isoform of spectrin involved and the subset of lymphocyte receptors affected.

Finally, the spectrin-expressing Jurkat cell lines were evaluated for their ability to be activated, as measured

Table 1. Stimulation of IL2 Generation by Jurkat T Cells (pg/ml/10^5 Cells)

Stimulus\Cell Line	No Stimulation	PMA+ Ionomycin	CD3+ CD28	CD28+ PMA+ Ionomycin
vector	46.9 ± 0.4	1811.4 ± 313.3	75.1 ± 0.9	3773.2 ± 283.5
βI_{N-5}	45.7 ± 1.2	102.8 ± 5.5	47.0 ± 0.4	221.6 ± 31.9
βI_{14-15}	45.8 ± 0.6	78.0 ± 2.9	46.3 ± 0.9	161.8 ± 31.6
$\beta I_{N-5,15}$	49.1 ± 1.2	1029.8 ± 404.6	99.3 ± 6.4	3828.1 ± 347.9

Mean ± standard error of the mean; n = 3 for each determination.

by IL2 secretion (Table 1). There were no differences in the resting levels of IL2 between any of the cell lines or in the vector alone controls. Conversely, and regardless of the method of stimulation, cell lines expressing either βI_{N-5} or βI_{14-15} demonstrated markedly diminished responsiveness. This blockage was absolute (100%) when cells were stimulated by methods that depend on receptor engagement (e.g., anti-CD3 and anti-CD28), consistent with the reductions in surface CD45 and CD3. However, an unexpected result was the almost complete block observed when cells were stimulated by pathways that act downstream of CD45 and CD3, such as via phorbol ester (PMA) and ionomycin ± CD28 (an accessory receptor augmenting T cell stimulation, Michel et al., 2001). While independent observations have noted that even the PMA activation pathway in lymphocytes is suppressed by the loss of CD45 (Czyzyk et al., 1998; Kishihara et al., 1993), levels of suppression in excess of 60% are difficult to attribute to the loss of CD45 alone. While it is likely that suppression of other surface receptors that may also be affected by the spectrin peptides might account for some of this unusual potency, it seems likely that the inhibitory βI spectrin peptides must also exert a dominant-negative effect on signaling processes downstream of the early T cell receptor signaling defect normally associated with a pure CD45 deficiency. These data thus reveal that transfected spectrin peptides are a novel and potent suppressor of lymphocyte activation that appear to act at more than one step along the signal transduction pathway.

Stability of CD45 at the Membrane Is Unaffected by βI Spectrin Peptides

Given that total cellular CD45 is unchanged with any of the spectrin peptides, significant alterations in its synthesis, degradation, or surface shedding can be excluded. It was therefore of interest to determine whether spectrin peptides were blocking the delivery of CD45 to the surface or enhancing the rates of CD45 internalization and/or recycling. In studies of α-Na,K-ATPase trafficking, βI_{N-5} was able to block transport early in the secretory pathway in MDCK cells (Devarajan et al., 1997). While we cannot rigorously exclude action at a pre-Golgi step, since our anti-CD45 antibodies do not detect incompletely glycosylated forms of CD45, we consider this unlikely given that the total levels of mature CD45 are unaffected by any of the peptides (Figure 4). We therefore measured the lifetime of surface CD45 using surface biotinylation (Figure 5). CD45 in cells expressing either the vector alone or $\beta I_{N-5,15}$ demonstrated similar half-lives (6.7 ± 4.7 and 5.2 ± 4.2 hr, respectively). Jurkat cells with the βI_{N-5} and βI_{14-15} transfectants demonstrated slightly lower rates of CD45 internalization ($t_{1/2}$ = 16.4 ± 27.7 and 14.4 ± 18.3 hr, respectively), although given the variability intrinsic to these measurements, these differences did not achieve significance. It should also be noted that the CD45 surface lifetime in Jurkat cells is comparable to its overall half-life in a T cell hybridoma line of 8–9 hr (Minami et al., 1991). We therefore conclude that the impact of the βI_{N-5} and $\beta I_{N-5,15}$ peptides is to impair the delivery of an intracellular pool of CD45 to the membrane, rather than to destabilize CD45 once it reaches the PM.

The Cytoplasmic Domain of CD45 Binds Ankyrin with High Affinity

While CD45 has previously been demonstrated to bind directly to spectrin (Lokeshwar and Bourguignon, 1992), it seemed unlikely that this interaction was playing a role in the dominant suppressive effects of the βI_{N-5} and $\beta I_{15,15}$ peptides, given that these peptides share no common sequence that might harbor a CD45 binding site. Conversely, both peptides would compete for spectrin- and ankyrin-mediated linkages between CD45 and the cortical actin skeleton or with the motors of microtubule-based transport. We therefore sought evidence that CD45 interacted directly with ankyrin. In PBLs and in Jurkat cells, ankyrin was precipitated by antibodies to CD45 even more so than by antibodies to spectrin. We therefore undertook to determine whether the cytoplasmic domain of CD45 (cd-CD45) bound to ankyrin directly. Measured by surface plasmon resonance (Figure 6), cd-CD45 bound ankyrin with high affinity (K_d = 4.3 ± 3.0 nM). Control experiments carried out with BSA demonstrated no affinity for cd-CD45.

Discussion

The results presented here demonstrate that βI spectrin, previously thought not to be present in lymphocytes, is in fact required for the efficient delivery of CD45 and CD3 to the lymphocyte surface, for T cell activation, and we postulate for the maintenance of CD45-rich microdomains on the lymphocyte surface. The mechanism of this requirement involves an ankyrin-mediated linkage between CD45 and spectrin. Although not directly demonstrated, it is presumed that βI spectrin links a CD45-ankyrin membrane complex to either the actin skeleton or to microtubule-based motility pathways, or both. These conclusions are supported by several lines of data: (1) PBLs and Jurkat T cells express multiple isoforms of spectrin, but βI spectrin is most consistently codistributed with CD45 in cytoplasmic aggregates and in surface polarized membrane patches and blebs; (2) Nonoverlapping βI spectrin peptides, representing either spectrin's ankyrin binding site or its actin/ARP1

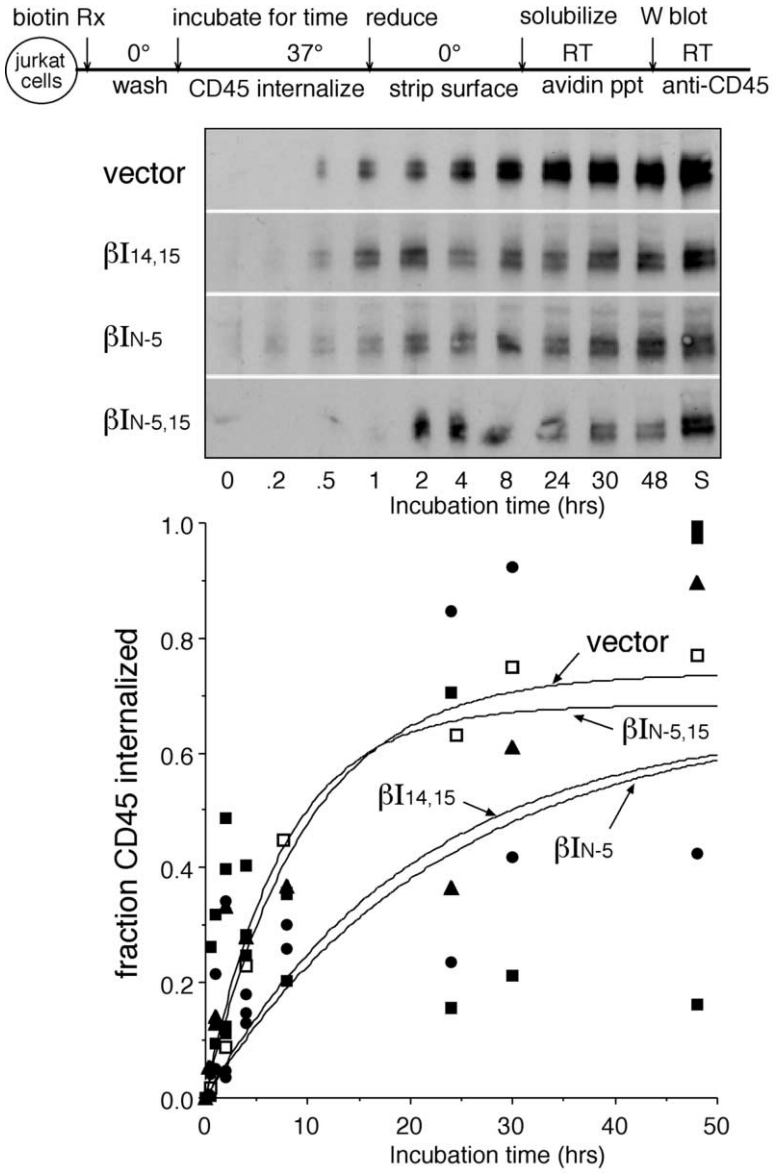

Figure 5. The Loss of Surface CD45 Is Not Due to Accelerated Endocytosis

(Top) To determine if the reduction in surface CD45 was caused by reduced rates of delivery versus enhanced rates of internalization, Jurkat cells expressing the various constructs were cooled to 0°, surface labeled with reducible biotin, and warmed to 37° for periods of time. At each time point, surface CD45 was determined by incubating the cells at 0° with reduced glutathione to liberate surface accessible biotin, followed by precipitation of biotinylated proteins with avidin and analysis by Western blotting with anti-CD45. (Bottom) Western blots from one experiment, demonstrating the increasing resistance of biotin-CD45 to reduction as it is internalized over time. These experiments were performed seven times. While there was variability between experiments, the trends within each experiment were clear and consistent. The results of five experiments with the most complete data across time are presented. Aggregated data from all experiments, fitted by nonlinear regression to a simple one-rate kinetic model, are represented by the curves. The fitted half-lives (hr) for surface CD45 from this analysis were: vector alone, 6.7 ± 4.7 hr; for βI_{N-5} cells, 16.4 ± 27.7 hr; for βI_{14-15} cells, 14.4 ± 18.3 hr; for $\beta I_{N-5,15}$ cells, 5.2 ± 4.2 hr. Note that there was no acceleration, and possibly even a reduction (albeit not of significance), in the internalization rate of surface CD45 in the presence of the βI_{N-5} and βI_{14-15} spectrin peptides.

binding site, both suppress the surface display of CD45 and CD3; (3) Suppression of CD45 is lost when these two sites are linked in a single peptide; (4) This effect is specific for βI spectrin and is not seen with βIII spectrin peptides; (5) Only a subset of lymphocyte receptors are suppressed; other surface proteins such as CD43 are unaffected; (6) Spectrin peptides that suppress CD45/CD3 lead to a near total loss of Jurkat T cell activation; (7) Spectrin peptides do not affect total cellular pools of CD45, excluding a direct action on cellular viability or protein synthesis; (8) The surface lifetime of CD45 is unchanged, and possibly even lengthened, in the presence of suppressive spectrin peptides; and (9) The cytoplasmic domain of CD45 binds directly both in vivo (as measured by coimmunoprecipitation) and in vitro (surface plasmon resonance) to ankyrin with high affinity. Taken with earlier data demonstrating the presence of cytoplasmic membrane aggregates involving spectrin and ankyrin that are rapidly recruited to the lymphocyte surface upon activation (Lee et al., 1988), and a similar cytoplasmic pool of CD45 that is also redistributed with T cell activation (Minami et al., 1991), these results reveal a novel role for the lymphocyte cytoskeleton in managing surface receptor display, organization, and signaling.

In other cells, the spectrin-ankyrin skeleton forms a complex multifunctional lattice that can bind a variety of cytosolic and membrane proteins and acidic phospholipids. Spectrin can also interact with all major cytoskeletal filament systems (actin, microtubules, and intermediate filaments, and thereby tether organized protein arrays to the cytoskeleton to form "linked mosaics" (De Matteis and Morrow, 2000). A corollary to this concept is the notion that spectrin and ankyrin, together with other adaptor proteins, may link subsets of transport vesicles to microtubule-based motors, and thereby facilitate the transport of such molecules through the secretory or endocytic pathways. This spectrin ankyrin

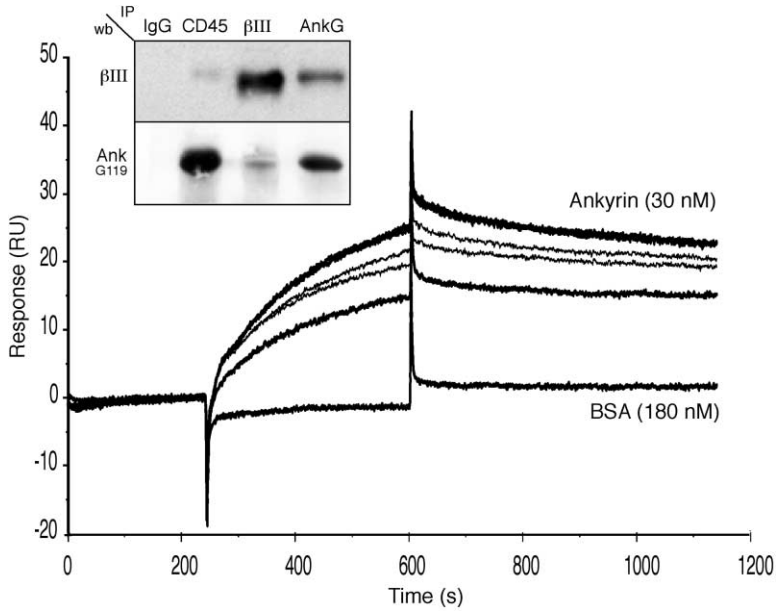

Figure 6. The Cytoplasmic Domain of CD45 Directly Binds Ankyrin

(Top) Immunoprecipitation of Jurkat cell lysates with antibodies to CD45, βIII spectrin, or Ank_{G119}, followed by Western blotting with antibodies to ankyrin, suggests a direct in vivo interaction between CD45 and ankyrin. This result is consistent with the direct interactions detected in peripheral blood leukocytes (Figure 1).

(Bottom) The cytoplasmic domain of CD45 (cd-CD45) was assayed for its ability to bind directly to ankyrin, as measured by surface plasmon resonance. Increasing concentrations of Ank_R or BSA were passed over immobilized cd-CD45. Binding constants were determined by nonlinear regression analysis, fitted to a bimolecular interaction model. While BSA demonstrated no affinity for cd-CD45, ankyrin bound avidly ($K_d = 4.3 \pm 3.0$ nM).

adapter protein tethering system (SAATS) model can explain the impact of inhibitory spectrin peptides on the transport of α-Na,K-ATPase from the ER to the Golgi (Devarajan et al., 1997), the disruption that such peptides cause to VSV-G transport (Godi et al., 1998), and the retrograde trafficking of axonal vesicles in the squid axon (Muresan et al., 2001). Other studies demonstrate that ankyrin is required in thymic, muscle, and nervous tissue for the proper intracellular sorting of voltage gated ion channels, Ca^{2+} ATPase, and the ryanodine (IP3) receptor (Tuvia et al., 1999; Zhou et al., 1998).

In lymphocytes, the organization of macromolecular microdomains centered around the CD3/TCR complex has emerged as a central and crucial theme in the control of T cell signaling (Leitenberg et al., 2001). Factors driving the organization and distribution of these complexes include interactions between the receptors themselves and with costimulatory molecules such as CD45 and CD4, interactions with the actin cytoskeleton as controlled by several kinases or small G-proteins (Bunnell et al., 2001; Yu et al., 2001), and differential partitioning into glycolipid-rich lipid microdomains (DIGs) (Balamuth et al., 2001; Ishii et al., 2001; Janes et al., 2000). We suggest that an additional factor controlling CD45 and CD3/TCR organization is the spectrin and ankyrin skeleton. We envision two similar but distinct roles for the βI spectrin-ankyrin lymphocyte skeleton. The first is its putative role in clustering, and presumably stabilizing, the perinuclear spectrin and CD45-rich lymphocyte aggregates (Figure 7A). Spectrin is proposed to link CD45- and CD3/TCR-rich trafficking vesicles emerging from the TGN or from late endosomal recycling compartments into a receptor-rich reservoir poised for recruitment to the PM. Spectrin and ankyrin tether this cargo to microtubule-based motors for transport to the PM (Figure 7B). These motors may be either dynein-dynactin (for minus-end directed transport, Holleran et al., 1996) or a member of the kinesin family (for plus-end directed transport, Takeda et al., 2000). Although we cannot exclude participation of spectrin and ankyrin in pre-Golgi steps along the secretory pathway, as suggested in our earlier work (Devarajan et al., 1997), the accumulation of fully glycosylated CD45 in the presence of inhibitory spectrin peptides indicates that the predominant blockage in CD45 trafficking must be post-medial Golgi.

The second role of the lymphocyte βI spectrin-ankyrin skeleton is to form defined microdomains involving CD45 and CD3/TCR (Figure 7C). By tethering these molecules into discrete clusters at the PM, we hypothesize that spectrin and ankyrin may prevent their uncontrolled diffusion into DIG rafts or into other microdomains, such as those defined by the interaction of CD43 with the actin skeleton via the adaptor protein moesin (Allenspach et al., 2001; Delon et al., 2001). With lymphocyte activation, we anticipate that CD3/TCR is released from the spectrin-ankyrin and CD45 complex, allowing its putative redistribution into DIG rafts (Figure 7D). Because the CD3/TCR dynamically associates with DIG rafts (Leitenberg et al., 2001), and because CD3 exposes only a very short peptide sequence to the cytoplasm, we postulate that the interaction of the CD3/TCR with spectrin and ankyrin is indirect, probably mediated by CD45. Spectrin βI peptides that abrogate the ankyrin-mediated interaction of CD45 with spectrin, either by creating transport complexes that lack an ankyrin binding domain (e.g., $\beta I_{N\text{-}5}$) or by directly saturating the spectrin binding domain on ankyrin (e.g., $\beta I_{14\text{-}15}$), dominantly suppress spectrin-mediated associations with the filamentous skeleton and the motors of transport (Figure 7E) and disrupt spectrin-ankyrin microdomains (Figure 7F).

Finally, it must be recognized that the interaction of spectrin and ankyrin with the lymphocyte membrane is obviously more complex than outlined in Figure 7. One surprising result of these studies is the spectrin-dependent inhibition of even receptor-independent activation pathways (Table 1). We believe that this effect derives from the involvement of spectrin not only in mediating the surface presentation of CD45 and CD3 but also from

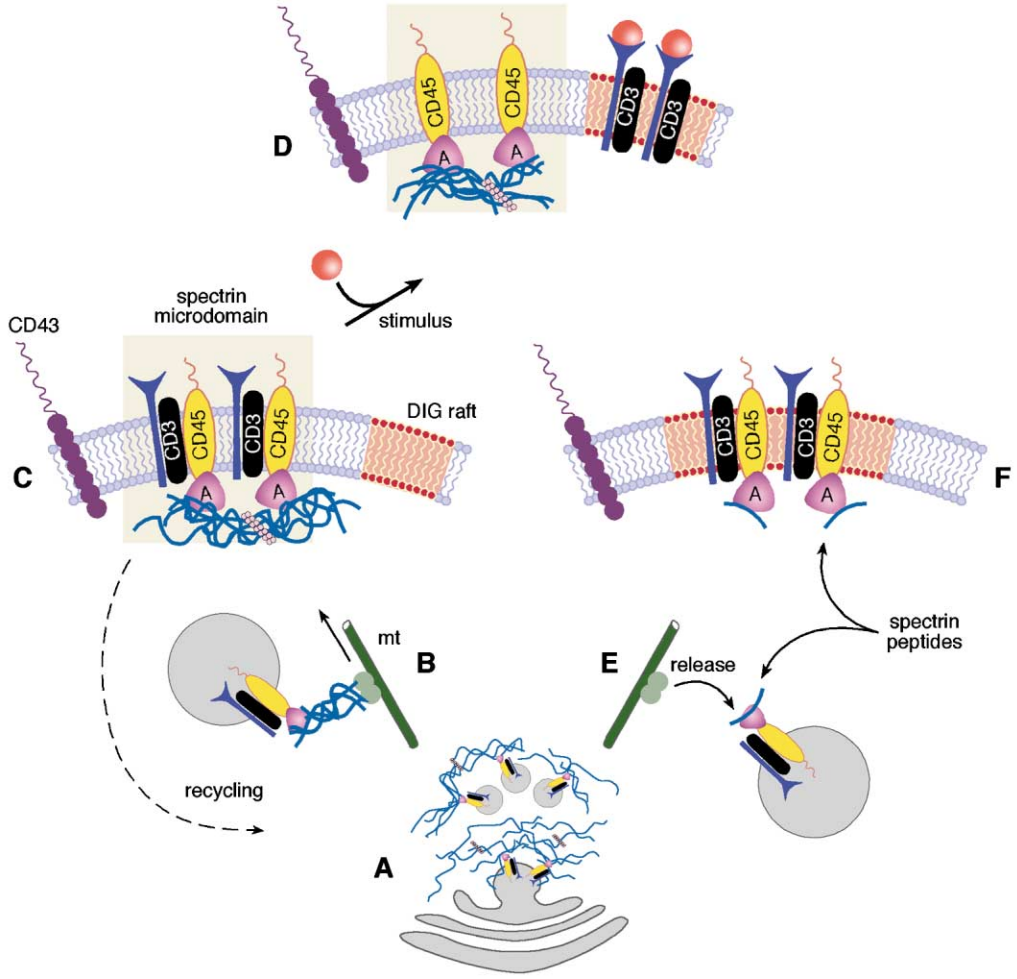

Figure 7. Hypothetical Model of How Spectrin and Ankyrin May Modulate CD45 Organization

(A) Vesicles exiting the trans-Golgi network (TGN) or recycled from late endosomal compartments are clustered by spectrin and ankyrin in the TGN region.

(B) Spectrin tethers CD45- and CD3/TCR-containing transport vesicles to motors of active transport (dynein-dynactin or kinesin family members) for transport to the PM along microtubules.

(C) At the lymphocyte membrane, spectrin and ankyrin organize CD45 and CD3/TCR into membrane microdomains. These domains are tethered to the cortical actin skeleton via spectrin. Other proteins such as CD43 do not participate in these interactions. These domains are distinct from detergent insoluble glycolipid rafts (DIG) that may harbor other costimulatory molecules.

(D) With activation, there is release of CD3/TCR from the spectrin-ankyrin and CD45 microdomain, with concomitant association with DIG rafts (and other costimulatory molecules).

(E) Inhibitory βI spectrin peptides block CD45 and CD3/TCR trafficking by competitively displacing the CD45- and CD3/TCR-containing vessels from the motors of microtubule transport.

(F) At the PM, the inhibitory spectrin peptides disrupt the organization of the spectrin-ankyrin microdomain, leading to abrogation of this domain. Spectrin peptides may also disrupt the organization of PKC (not shown), adding to its profound inhibitory effects on T cell activation.

its established role in mediating the compartmentalization of PKC in activated lymphocytes (Gregorio et al., 1992, 1994). There is a tight association of PKC with a particulate spectrin and ankyrin complex after T cell activation. While not directly studied, we anticipate that the inhibitory β spectrin peptides reported here will also decouple the association of PKC from this particulate complex and thereby possibly disrupt its activity. In future studies, it will thus be interesting to evaluate whether the selective abrogation of spectrin's PKC binding activity can suppress downstream activation events without disturbing surface CD45 or CD3 levels. We also do not understand why only the βI isoform of spectrin modifies the distribution and display of CD45 and CD3. In preliminary studies, we have noted that additional surface molecules beyond CD45 and CD3 are affected by βI spectrin peptides, although many proteins (such as CD43) are not. Whether different spectrins and ankyrins are needed to organize other receptors, or conversely whether a given receptor is associated with different spectrins and ankyrins depending on its location in the cell, remain questions for future study. Earlier work has also reported a direct interaction between CD45 and spectrin, presumably βII spectrin (Lokeshwar and Bourguignon, 1992). We do not know if βII spectrin, which is widely distributed on the PM, plays a role in

the delivery or stabilization of CD45. However, since the phosphatase activity of CD45 can be regulated by its interaction with spectrin (Lokeshwar and Bourguignon, 1992), and since all spectrins will coprecipitate with CD45 to some degree (Figure 1), it seems likely that spectrin (and ankyrin) will have many roles in the lymphocyte beyond those explored here. The present results nevertheless clearly establish the central importance of spectrin and ankyrin for the establishment and maintenance of CD45 and CD3 receptor domains in T lymphocytes, and a role for spectrin-ankyrin in mediating the signal transduction events central to lymphocyte activation.

Experimental Procedures

Preparation of Spectrin Constructs and Transfected Cell Lines

βI spectrin peptides were expressed from existing βI spectrin constructs (Devarajan et al., 1997). Stable Jurkat lines were established by limiting dilution in G418 after transfection using DMRIE-C (GIBCO-BRL). In brief, 6 μl of DMRIE-C, 1.0 ml OPTI-MEM I, and 2–4 μg DNA was incubated at room temperature for 45 min. Cells (10^6) were added in 0.2 ml serum free medium and incubated at 37° for 4 hr, after which 2 ml RPMI with 15% FBS and 50 ng/ml PMA was added. Selection in G418 (1.5 mg/ml) was initiated after 24 hr. At 48 hr, cells were diluted into 96-well plates and grown in RPMI and 10% FBS containing 1.5 mg/ml G418.

RT-PCR Analyses

Primers specific to βI spectrin were used to amplify RNA from either Jurkat cells or human PBLs. A common forward primer in exon 30 (5′-TGAGAAGTCCACGGCCAGCTGGGCA -3′ or 5′-TGTGCCAGTT CTCGAGGGAT-3′) was used with isoform specific reverse primers to differentiate between spectrin βIΣI (5′-GCGGATCCCTAGTAGGG GTGAGAGGG-3′) and spectrin βIΣII (5′-GCGGATCCCTACTTCTTT TTGGG-3′). Spectrin βIII was amplified using forward primer 5′-ACA CCACAGACAAGTTCCGCTT-3′ and reverse primer 5′-AGCGTTTT TCTCGCTCTCGT-3′. Construct expression was verified by RT-PCR. For $\beta I_{N\text{-}5}$: forward primer 5′-AGCTGCCACCATGGACTACAAGGAC GACGATGACAAGGGACCTA-3′ (with the FLAG epitope sequence), reverse 5′- TTCTGAACAACTTCCCGCTC -3′; for $\beta I_{14\text{-}15}$: forward as for $\beta I_{N\text{-}5}$, reverse 5′-GGCATTCACATTGTCCACCCGC-3′; for $\beta I_{N\text{-}5,15}$: forward 5′-GGCCCAGGCGGACCTGCGTCAG -3′, reverse 5′-GGC ATTCACATTGTCCACCCGC-3′. βIII spectrin constructs were generated as before (De Matteis and Morrow, 2001). Spectrin $\beta III_{N\text{-}5}$ included codons 1–851; $\beta III_{14\text{-}15}$ codons 1696–1906; $\beta III_{N\text{-}5,15}$ was a chimera of these two.

Isolation of Primary Lymphocytes

Primary lymphocytes were isolated by centrifuging human leukocytes over Lymphocyte Separation Medium (ICN Biochemicals). Samples were obtained under protocol as Leukopaks from the Yale-New Haven Hospital blood bank.

Antibodies

Pabs included: RAF-A, αII spectrin; MUS1, βIΣII spectrin; RAS-C and βI_f, αI and βI spectrin, respectively; 10-D, βII spectrin; $\beta III_{7\text{-}11}$, βIII spectrin (Stankewich et al., 1998); ANK-R, erythrocyte ankyrin; and Jasmin, $AnkG_{119}$. Mab C19, to βIΣ1 spectrin, was a gift from V. Marchesi (Yale). Commercial antibodies included anti-CD45 (HI30, Pharmingen); anti-CD3 (UCHT1, Pharmingen), and anti-CD43 (Caltag). FACS analysis utilized a Becton Dickinson FACS Sort. Intracellular staining of FLAG-labeled peptides was done in presence of 0.3% saponin after fixation in 1% paraformaldehyde (Gomez et al., 2000).

Immunofluorescence

Cells were settled onto poly-lysine-coated glass coverslips or multichamber slides and fixed 20 min in cold acetone:methanol (1:1); rinsed 3× with PBS, and blocked 1 hr in 10% BSA (w/v). Antibodies were applied overnight at 4°. Secondary antibodies were incubated 1 hr at room temperature. Slides were viewed using an Olympus AX-70 or an IX-70 confocal microscope. Volume deconvolution was performed on images collected at 0.5 or 1.0 μm Z-intervals with Openlab (Improvision, Inc.).

IL-2 Secretion Assays

Cells (10^5) were maintained 48 hr in G418 free medium prior to assay, then stimulated for 24 hr with: 80 nM PMA, 2 μM ionomycin; 1 μg/ml anti-CD3 and anti-CD28; or 1 μg/ml anti-CD28, 80 nM PMA, 2 μM ionomycin. Cell supernatants were assayed for IL-2 by ELISA kit (Pierce-Endogen). All determinations were done at least three times and the results averaged.

Immunoprecipitation

Lysates were prepared in RIPA lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% NP-40, and 0.5% desoxycholate) at 4°, precleared, incubated overnight with antibodies, and precipitated 1 hr at 4° with Protein A-Sepharose.

Surface Biotinylation

Cells (2×10^6/ml in PBS) were incubated with 25 μg/ml NHS-S-biotin (Pierce) for 15 to 30 min on ice. Biotinylation was quenched in 10 mM Tris (pH 7.4), 140 mM NaCl. After timed incubations at 37°, surface accessible biotin was reduced by glutathione. Biotinylated species remaining in RIPA lysates were bound to streptavidin beads, separated by SDS-PAGE, and CD45 detected by immunoblotting. Because surface levels of CD45 in the $\beta I_{N\text{-}5}$ and $\beta I_{14\text{-}15}$ transfected lines were reduced, biotin incubation times and the exposure times for these samples were lengthened to compensate for their reduced signal intensity.

Surface Plasmon Resonance

cd-CD45 (aa 564 to 1268), prepared as a GST fusion protein, was a gift from Frank Jirik. GST was removed with thrombin, and the peptide immobilized on a CM5 sensor chip (BIAcore) (Pradhan et al., 2001). Human erythrocyte ankyrin (AnkR) was in PBS, and K_d was calculated by nonlinear least squares fitting to a bimolecular binding equation using BIAevaluation software 3.0.

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